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Role of Glutathione Peroxidase in Rheumatoid Arthritis: Analysis of Enzyme Activity and DNA Polymorphism

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ABSTRACT

Aberrant expression of the antioxidant enzyme glutathione peroxidase (GPx) could contribute to the etiology of rheumatoid arthritis (RA). However, previous enzyme activity studies examining this relationship were inconclusive. Indirect evidence for this relationship derives from the known efficacy of gold therapy in RA, since gold compounds specifically inhibit GPx. The hypothesis that variants of GPx are associated with RA was examined by two approaches: enzyme activity analysis and restriction fragment length polymorphism (RFLP) association analysis. No significant difference was found in whole blood GPx activity between 28 RA patients and 36 controls. GPx activity appeared to be independent of sex, race, or type of drug treatment. However, a statistically significant difference was found with respect to treatment responsiveness. RA patients classified as good responders to gold therapy, but who were no longer taking gold, had a significantly higher GPx activity compared to both the controls and good responders currently on gold therapy. Aberrantly high GPx activity could contribute to RA by generating excess oxidized glutathione, a potent collagenase activator. Gold therapy would reduce GPx activity to normal levels. The restriction enzyme *Pvu* II in conjunction with a GPx gene probe identified a useful RFLP (A1, 22 kbp; A2, 15 kbp) with allelic frequencies of A1 and A2 equal to 0.11 and 0.89, respectively, in the control population. No statistically signficant association, however, could be demonstrated between this allelic variant of the GPx gene and RA.

INTRODUCTION

RHEUMATOID ARTHRITIS (RA) has a multifactorial polygenic etiology. A variety of initiating factors trigger a local immune resonse in the RA synovium. The formation of immune complexes activates a cascade of chemical mediators of inflammation, reactive oxygen species (ROS), and collagenases, ultimately leading to RA in genetically susceptible individuals (Schumacher and Gall, 1989). Part of the genetic susceptibility is accounted for by genes within the major histocompatibility complex (MHC). However, formal genetic studies suggest the total genetic contribution of the MHC to RA is only 37% (Deighton *et al.*, 1989); what accounts for the remaining 63% is unknown. *In vivo* and *in vitro* studies have suggested ROS to be of pathogenic importance in RA. Elevated levels of peroxides have been found in RA serum and synovial fluid (Lunec *et al.*, 1981; Biemond *et al.*, 1986). On exposure to ROS, hyaluronic acid in synovial fluid is depolymerized and loses its lubricating properties (Blake *et al.*, 1981).

The glutathione redox system is a major defense mechanism against ROS. It consists of the following enzymatic reactions:

$$H_2O_2 + 2GSH \xrightarrow[GPx]{} 2H_2O + GSSG$$

$$GSSG + NADPH + H^+ \xrightarrow[GR]{} 2GSH + NADP^+$$

Reduced glutathione (GSH) is utilized by glutathione peroxidase (GPx) to reduce H_2O_2, or other peroxides, to nontoxic forms. The oxidized glutathione (GSSG) resulting from this reaction is then reduced by glutathione reductase (GR).

There is evidence suggesting that the glutathione redox system is an important defense mechanism against ROS released by the activated immune cells in RA joints. Human rheumatoid synovial fluid contains no significant superoxide dismutase or catalase activity (Blake *et al.*, 1981); furthermore, cartilage proteoglycans in synovial medium

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were only minimally protected by these enzymes (Klamfeldt and Marklund, 1987). Levels of GSH in joint tissue and erythrocytes decrease during active RA disease (Munthe *et al.*, 1981, 1986). The activity of enzymes that protect against ROS damage, such as GPx, has been suggested to be insufficient to deal with the increased peroxides in RA (Lunec *et al.*, 1981; Biemond *et al.*, 1986).

Investigations concerning GPx activity in RA have yielded conflicting results. Most studies have found a significantly depressed GPx level (Munthe *et al.*, 1986; Tarp *et al.*, 1987; Vanella *et al.*, 1987; Imadaya *et al.*, 1988), or a GPx level comparable with controls (Peretz *et al.*, 1987; Borgland *et al.*, 1988; Clerc *et al.*, 1988; Abella *et al.*, 1990) in the hemolysates and various blood components during the active phase of RA. Two investigators found increased levels. Mezes *et al.* (1987) observed an increased erythrocyte GPx activity in 15 female RA patients compared to controls, but no increase in males. Braven *et al.* (1989) reported a 30% increase in erythrocyte GPx activity in 43 RA patients when compared to 29 healthy controls.

There are several lines of reasoning to link either aberrantly low or high GPx levels with the etiology of RA. As suggested above, low levels of GPx activity may be insufficient to deal with the increased peroxides found in RA joints (Lunec *et al.*, 1981; Biemond *et al.*, 1986). On the other hand, aberrantly high levels of GPx in RA could explain the efficacy of the second-line drugs, penicillamine and gold. These drugs are potent inhibitors of GPx (Chaudiere and Tappel, 1984; Chaudiere *et al.*, 1984). In situations of oxidative stress, GPx produces high levels of GSSG, which is a potent activator of leukocyte collagenase (Tschesche and Macartney, 1981).

In the studies reported here, the role of GPx in RA was examined first on the enzyme level with reference to population variables including sex, race, and medication. It was further examined on the gene level by restriction fragment length polymorphism (RFLP) association analysis.

MATERIALS AND METHODS

Populations

Thirty-six healthy individuals (mean age 45 ± 18 years) and 28 RA patients (mean age = 55 ± 10 years) from the Philadelphia area were studied. All RA patients were positive for rheumatoid factor and met the American Rheumatism Association criteria for definitive RA (Ropes *et al.*, 1958). The average duration of disease was 14 ± 8 years. All patients were taking a nonsteroidal antiinflammatory drug. Twenty two patients were receiving one second-line treatment as listed in Table 1. RA patients were also classified according to their response to gold therapy. Good gold responders were those individuals presently being treated or previously treated with gold for at least 1 year, who had no ill side effects, and indicated relief from arthritic symptoms. Poor responders were on gold therapy at least 6 months with no symptomatic improvement. The race and sex distribution of the patient population was 32% black males, 32% white males, 12% black females, and 24% white females, and that of the controls was 17% black males, 14% white males, 44% black females, and 25% white females.

Table 1. Mean Glutathione Peroxidase Activity in Controls and Rheumatoid Arthritis Patients with Variations in Drug Treatment

	N	Glutathione peroxidase *activity*[a]
Controls	36	15.83 ± 5.2
Rheumatoid patients	28	16.33 ± 6.6
Drug treatment[b]		
Methotrexate	9	17.09 ± 5.8
Gold compounds[c]	9	13.54 ± 2.7
Hydroxychloriquine	3	12.34 ± 2.1
Penicillamine	1	11.90
Prednisone alone	3	18.60 ± 6.8
Prednisone and 2nd line drug above	10	14.43 ± 3.1

[a] μmoles of NADPH oxidized/min • gram of Hb.
[b] 10 of the 22 individuals taking one of the various second-line drugs were also taking prednisone.
[c] Gold sodium thiomalate (7), Aurothioglucose (2).

Glutathione peroxidase assay

GPx was assayed in whole blood by a modification (Thomson *et al.*, 1977) of the coupled assay of Paglia and Valentine (1967). The hemolysate was prepared by diluting 0.5 ml of blood to 1.0 ml with isotonic saline solution, adding an equal volume of double-strength Drabkin's reagent, and four volumes of 0.02 *M* phosphate buffer (pH 7) containing 0.5% Triton X-100. The incubation mixture (2.7 ml) contained: 2 μmoles of glutathione, 0.5 units of glutathione reductase, 1 μmole of NaN_3, and 0.1 μmole of NADPH in 20 m*M* phosphate buffer (pH 7), with 6 m*M* EDTA. To assay for GPx, 0.3 ml of the hemolysate was added to the incubation mixture, and the reaction was initiated by adding 10 μl of 30 m*M* *t*-butyl hydroperoxide. The conversion of NADPH to $NADP^+$ was followed by monitoring the change in absorbance at 340 nm for 4 min. Hemoglobin (Hb) was determined quantitatively using the Sigma diagnostic kit for total Hb (St. Louis, MO). Units of GPx were reported as μmoles of NADPH oxidized/min/gram of Hb.

RFLP analysis

High-molecular-weight DNA was isolated from peripheral blood samples by standard proteinase-K treatment and phenol-chloroform extraction techniques as described by Bell *et al.* (1981). DNA (7 μg) from at least 10 individuals was cleaved with one of 13 different restriction endonucleases (*Bam* HI, *Bgl* I, *Bste* II, *Eco* RI, *Eco* RV, *Hin*d III, *Kpn* I, *Pst* I, *Pvu* II, *Sal* I, *Taq* I, *Xba* I, *Xho* I) under conditions recommended by the suppliers. The DNA fragments were electrophoresed on 1% agarose gels and transferred to Zeta-probe nylon membranes by the alkaline or Southern procedure. The membranes were then probed with ^{32}P-labeled GPx cDNA and washed according to conditions recommended by the manufacturer of Zeta-probe

(Bio-Rad, Richmond, CA). A useful RFLP was found for the *Pvu* II restriction enzyme. The remaining controls and RA patient DNA samples were then screened in the same way for the presence of the GPx gene *Pvu* II RFLP.

RESULTS

GPx activity in RA patients and controls

GPx activity in whole-blood hemolysates was compared between 28 RA patients and 36 healthy controls. No significant difference was found between these two groups (Table 1). There were also no differences in GPx activity when these groups were divided according to race (data not shown). In both the control and RA groups, GPx activity was greater in the females (RA females = 17.71 ± 8.5, control females = 16.35 ± 4.6) than in the males (RA males = 15.43 ± 5.2, control males 14.64 ± 6.6); however, the difference found is not statistically significant.

As also shown in Table 1, 22 of the 28 RA patients were receiving second-line drug treatment, and 10 patients were also taking prednisone. The GPx activity of each of these groups was compared against the controls and no differences were found.

Gold compounds have multiple cellular effects that include irreversible inhibition of GPx activity (Chaudiere and Tappel, 1984; Chaudiere *et al.*, 1984). It is known that RA patients respond differently to gold therapy. It may be that the individuals who respond to gold have intrinsic differences in their GPx. This was first examined by separating the RA group into poor and good responders and examining their GPx activity. Of the 28 RA patients studied, 26 could be classified as good or poor responders. No difference was found in the GPx activity between these two groups (Table 2).

However, good responders were more likely to be taking gold, and since gold inhibits GPx activity, it may be masking any intrinsic difference that exists. Therefore, the good responder group was separated into those individuals who were currently taking gold and those who were not. Those good responders currently off gold therapy had a significantly higher GPx activity than those on gold therapy. Their GPx activity was also significantly higher than that of the controls.

RFLP analysis

A useful RFLP was found with the *Pvu* II restriction enzyme, which revealed a polymorphic allele, designated A1. In all cases, A1 (22 kbp) was found in a heterozygous form; the normal allele (A2) is apparently 15 kbp. The allelic frequencies of A1 and A2, in the control population, are 0.11 and 0.89, respectively. That is eight copies of A1 were found in 36 chromosomal pairs. Figure 1 shows sample lanes of control DNA cleaved with *Pvu* II and probed with GPx cDNA. The 22-kbp RFLP is shown in lane 1. The frequency of this allele was then compared between the control and RA patients. The data is summarized in Table 3. Although there appears to be threefold higher frequency of the A1 allele in the controls compared to the RA patients, this difference was not statistically significant. Again, there were also no statistical differences observed between blacks and whites or males and females.

TABLE 2. MEAN GLUTATHIONE PEROXIDASE ACTIVITY IN RHEUMATOID ARTHRITIS PATIENTS WITH RESPECT TO GOLD THERAPY RESPONSE

	N	Glutathione peroxidase *activity*[a]
Controls	36	15.83 ± 5.2
Poor responders	9	15.41 ± 3.5
Good responders	17	17.13 ± 8.2
on gold therapy	9	13.53 ± 2.7
off gold therapy	8	21.18 ± 10.4[b,c]

[a] μmoles of NADPH oxidized/min • gram of Hb.
[b] $p = 0.05$ when compared to good responders on gold.
[c] $p = < 0.05$ when compared to control group.

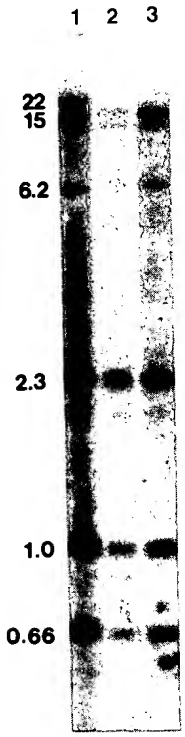

FIG. 1. Characteristic lanes from a Southern blot of *Pvu* II-cleaved control DNA probed with GPx cDNA. Lane 1 is apparently heterozygous for the A1 (22 kbp) and A2 (15 kbp) alleles; lanes 2 and 3 are homozygous for A2.

DISCUSSION

The hypothesis that variants of the glutathione redox enzyme, GPx, is associated with RA was examined. First, GPx activity was measured in an RA patient group of an appropriate size (≥28) to detect a 20% change in GPx activity from that of the controls at the level of $p \leq 0.05$. No

TABLE 3. PRESENCE OF THE A1 ALLELE OF THE GPx *Pvu* II RFLP IN CONTROLS AND RHEUMATOID ARTHRITIS PATIENTS[a]

	Controls			*Rheumatoid arthritis*			
	Male	*Female*	*Subtotal*	*Male*	*Female*	*Subtotal*	*Totals*
Black	2 (6)	4 (16)	6 (22)	1 (8)	0 (3)	1 (11)	7 (33)
White	0 (5)	2 (9)	2 (14)	1 (8)	0 (6)	1 (14)	3 (28)
Totals	2 (12)	6 (25)	8 (36)	2 (16)	0 (9)	2 (25)	

[a]Total number sampled are in parentheses.

statistically significant difference was found. This suggests that GPx variation may not be a contributing factor to the etiology of RA. It is consistent with other reports that have found no difference in the GPx activities between the RA and control populations (Peretz *et al.*, 1987; Borgland *et al.*, 1988; Clerc *et al.*, 1988; Abella *et al.*, 1990). It may also be that the difference in GPx that contributes to the etiology of RA is not detectable by simple enzyme activity analysis of whole blood lysates. However, using a similar assay, Braven *et al.* (1989) found a 30% increase in GPx activity in statistically appropriate groups of RA patients compared to controls. The difference between our results and Braven's may have to do with the mixed populations used in both.

A frequently overlooked but important variable, which may mask any differences in GPx enzyme activity between RA and control populations, is medication. Specifically, the second-line drugs (gold salts and penicillamine) are known to irreversibly inhibit GPx activity *in vitro* (Chaudiere and Tappel, 1984; Chaudiere *et al.*, 1984) and *in vivo* (Dillard and Tappel, 1986). To demonstrate the effects of medications on GPx activity, RA patients were categorized by the type of medications they were taking at the time of sampling. GPx activity of the RA patients taking the various drug classes was compared with the controls. Although no statistically significant difference was found, the GPx activities of RA patients taking either gold or penicillamine were lower by at least 13% from that of the controls.

This result agrees with the only published reports that examine the effects of specific second-line drugs. Abella *et al.* (1990) concentrated their studies on the effects of penicillamine and found no difference in the protein concentration of GPx as detected by radioimmunoassay between RA patients treated or not treated with penicillamine and controls. This radioimmunoassay, however, would presumably detect GPx in both active and inactive forms, masking any inhibitory effects of the drugs. Similar to the present study, Braven *et al.* (1989) divided their RA population into groups based on the drugs being taken. They, too, found no statistically significant differences in the activity of GPx between individuals taking gold, penicillamine, or nonsteroidal antiinflammatory drugs, and their controls.

Because gold inhibits GPx *in vivo*, a detectable difference would be expected. The explanation for why no difference was found in both this study and Braven *et al.* (1989) could be the small sample sizes (<10 individuals). Alternatively, the expected difference may not have been observed because in the absence of the drugs, GPx activity is higher in RA patients, and treatment serves to lower the activity to normal levels.

Thus, one further variable studied here was response to gold therapy. Not all RA patients respond to gold therapy. Since gold compounds inhibit GPx, it may be that the individuals who respond to gold have an intrinsic difference in their GPx. No difference in GPx activity was found between the good gold responders and the poor gold responders. However, most of the good responders were on gold, and since the drug inhibits GPx, it may be masking any intrinsic difference in GPx activity. Therefore, the good responder group was separated into those individuals who were currently on gold *versus* those no longer on the drug. A statistically significant difference was found between these two groups: good responders not on gold therapy had a significantly higher GPx activity than controls or RA patients on gold. The result suggests that a subset of RA patients (good gold responders) may have GPx of intrinsically higher activity. It has been speculated that increased GPx activity in RA patients may increase GSSG, a potent activator of collagenase, and thus indirectly lead to destruction of joint tissue (Chaudiere, 1986). Gold may work by inhibiting this hyperactive GPx.

Although this finding that GPx activity is significantly higher in good responders not on gold is unique, it must be accepted with caution. The result may reflect possible increases in GPx activity from the effects of the other drugs taken by the good responders off gold therapy. Five of these individuals were taking methotrexate, which had a higher GPx activity compared to the controls, although this difference was not statistically significant. Our conclusion may also suffer from a simple type I error of probability from the effect of too many analyses. The relatively wide variation in the GPx enzyme activity of RA patients off gold therapy (50%) is presumably due to the pharmacokinetics of gold. The plasma half-life of gold compounds may be as long as weeks to months in individuals on prolonged therapy. The length of time the gold-responsive RA patients were off gold was not recorded.

The difficulties in controlling variables such as drug treatment that affect enzyme activity analysis prompted us to examine the role of GPx variants in RA on the DNA level. No statistically significant linkage could be demonstrated between an allelic variant of GPx revealed by the

Pvu II RFLP and the occurrence of RA. This linkage should probably be reexamined in a larger group of patients. In any case, our data do not rule out the possibility that there may be alterations in nucleotide sequences that affect GPx regulation or structure that are not detectable with this particular RFLP. It should now be feasible to examine the regulatory and coding regions directly by amplification of the GPx DNA from selected populations followed by comparison of their sequences. For example, comparison of the gold responders, nonresponders, and controls might identify the basis for the differential GPx enzyme activity.

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